Q-524: Chemically defined medium for *Desulfovibrio vulgaris* stress studies and biomass production

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VIMSS Virtual Institute of Microbial Stress and Survival

DOE GENOMICS:GTL ACCELERATING DISCOVERY FOR ENERGY AND ENVIRONMENT
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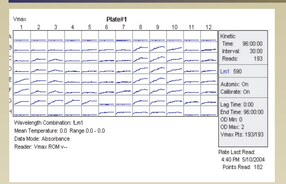
Abstract

A defined medium for optimal growth and maximum reproducibility of *Desulfovibrio vulgaris* was developed for biomass production for stress response studies. The medium was optimized by evaluating a variety of chemical components, including the removal of yeast extract, excess sulfate, and Fe, and redox conditions to optimize cell density and generation times, and to reduce lag times. Growth was monitored using direct cell counts, optical density, and protein concentration. The generation time for *D. vulgaris* in the original Baar's medium was 3 h, reaching a maximum density of 10^8 cells/ml and 0.4 OD600 nm. The newly developed medium, lactate-sulfate defined medium, version 4 (LS4D), supplies 0.06 M sodium lactate and 0.05 M sodium sulfate. Both ATCC-prepared Wolfe's vitamins and laboratory-prepared Thauer's vitamins were tried. It was determined that use of Wolfe's vitamins caused a >15 h increase in the lag phase, though no change in the generation time. Three differences were observed in the formulations: (1) Thauer's vitamins have 10x the concentration of Vitamin B12, (2) Thauer's 10X vitamin stock also includes 2 g choline chloride /l , and (3) Thauer's vitamins were prepared in our laboratory. Further LS4D medium includes tungsten, selenium, and copper as trace minerals. The generation time for *D. vulgaris* on LS4D was 5 h, with a maximum cell density of 10^9 cells/ml and a 0.9-1.0 OD600 nm. The reduction of $FeCl_2$ to 12.5mg/l (~60 uM) as a trace mineral reduced precipitates in the medium without affecting growth rates or generation times significantly. LS4D is well suited for the monitoring protocols, as well as the equipment and large scale processing needed for biomass production. Several reductant use protocols were tested, including titanium citrate, cysteine HCl, and sodium hyposulfite. It was found that more reproducible cultures and shorter lag time could be achieved by inoculating non-reduced medium (no reductant) with 10% actively growing mid-log phase culture. The mid-log phase culture had adequate reducing power in the fresh media to lower the redox sufficiently to allow for continued growth of the cells.

Background

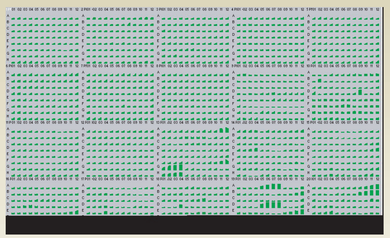
The Virtual Institute for Microbial Stress and Survival (VIMSS) is using a unique new multidisplinary approach to study the stress response pathways in metal reducing bacteria. Integrating ecogenomics, phenomics, transcriptomics, proteomics, metabolomics, fluxomics, and bioinformatics, VIMSS is developing high throughput techniques to determine stress responses with remarkable new insights. The ultimate goal of the project is to understand and apply these data to the natural and accelerated bioremediation of DOE sites contaminated with nuclear production legacy waste- notably uranium. The initial focus is to define the basic biology (phenomics) of *Desulfovibrio vulgaris*, an 'obligate' anaerobe, sulfate-reducer. In order to determine the minimum inhibitory concentrations (MIC) of a number of stressors, we developed a completely defined medium. The LS4D (Lactate Sulfate version 4 Defined) was developed for *D. vulgaris* biomass production to maximize reproducibility for all the other studies on the same cells, i.e. transcriptomics, proteomics, metabolomics, and fluxomics. Growth curves defining the physiological response of *D. vulgaris* to oxygen, nitrite, nitrate, and osmotic stresses were tested. Stress and inhibitory ranges of concentrations of these contaminants were measured as retardation or inhibition of growth of *D. vulgaris* using automated techniques.

Media and Methods

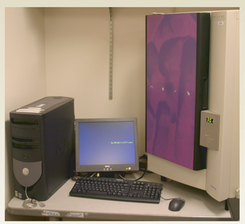


High throughput kinetic screening of microbial growth in a 96 well format. Plate reader put in incubator with each plate thermally sealed anaerobically in a bag and read at 15 min intervals for up to 200 h. Using SoftMax Pro 4.6 (Molecular Devices).

***Desulfovibrio vulgaris* Hildenborough (ATCC 29579). Cultures were stored at -80°C until used and were less than 3 transfers from the original culture from ATCC.**



Phenotypic Microarray Omnilog System™ (Biolog) for the simultaneous and rapid screening of the effects of hundreds of separate growth conditions. An array of metabolites, inhibitors, osmotic and ion effects as well as pHs can be tested. This system will be used to compare metabolic curves of mutant strains against the wildtype strain and to do automated growth curves for a variety of substrates and stressors.



Media Comparisons

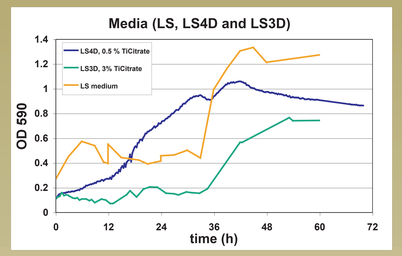
LS4D Medium

Compound	Molarity	ml/L stock	Supplier	Catalog #	Molarity in Medium	Storage
Degassed ddwater		810 ml				Anaerobic
Sodium sulfate	0.5M	100 ml	Fisher	S421-500	50 mM	Anaerobic
Sodium lactate	2M	30 ml	Fisher	S326-500	60 mM	4°C
Magnesium chloride	1M	8 ml	Fisher	M33-500	8.0 mM	Anaerobic
Ammonium chloride	4M	5 ml	Fisher	A687-500	20 mM	Anaerobic
Potassium phosphate	1M	2.2 ml	Fisher	P290-500	2.2 mM	Anaerobic
Calcium chloride	1M	0.6 ml	Fisher	C79-500	0.6 mM	Anaerobic
Thauers vitamins	10X	1 ml	Stock below			-20°C
Trace minerals		12.5 ml	Stock below			4°C
PIPES	1M	30 ml	Fisher	BP304-500	30 mM	Anaerobic
Resazurin (0.1%)	0.1%	0.16 ml	Sigma	R7017	0.016 µM	Anaerobic
Sodium Hydroxide (Used to adjust pH of medium to 7.20)	5N	~ 1.5 ml	Sigma	S8045	10 mN	
Titanium Citrate (Added to medium before inoculation)		5 ml	Stock below			

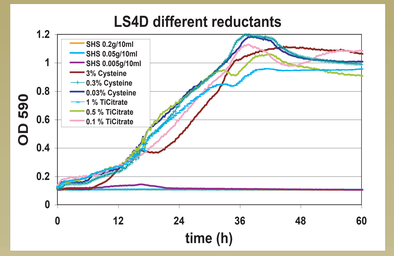
A variety of amendments to the traditional Baars and Lactate Sulfate medium (1) resulted in a defined medium LS4D (Lactate Sulfate Defined version 4) for *D. vulgaris* biomass production for stress response studies. The medium was optimized by evaluating a variety of chemical components, including the removal of yeast extract, excess sulfate, and Fe, testing different reductants, using different concentrations of lactate to optimize cell density (10^9 cells/ml) and generation times (5 hours), and to reduce lag times seen with other LS media.

	BAARs	LS	LS3D	LS4D
Sulfate	18 mM	50 mM	50 mM	50 mM
Lactate	30 mM	60 mM	60 mM	60 mM
Yeast	1g/L	1g/L	0	0
Vitamins	0	Thauers	Wolfes	Thauers
Minerals	0	Trace Minerals	Trace Minerals 3	Trace Minerals 3
Iron	0.1% $Fe(NH_4)_2(SO_4)_2$	0.1 g/L	0.1 g/L	0.1 g/L
Additional	$MgSO_4$ 2g/L $CaSO_4$ 1g/L	$MgSO_4$: 8mM HEPES	PIPES	PIPES

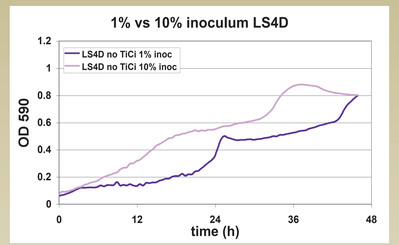
Trace Minerals	g/L	Supplier	Catalog #	Final Molarity of stock
Nitrilotriacetic acid	12.8	Sigma	N-0253	50 mM
$FeCl_2 \bullet 4\ H_2O$	1.0	Sigma	F2130	5.0 mM
$MnCl_2 \bullet 4\ H_2O$	0.5	Fisher	M87-100	2.5 mM
$CoCl_2 \bullet 6\ H_2O$	0.3	Fisher	C371-100	1.3 mM
$ZnCl_2$	0.2	Sigma	Z4875	1.5 mM
$Na_2MoO_4 \bullet 4\ H_2O$	0.05	Sigma	M1003	210 µM
H_3BO_3	0.02	Fisher	BP168-500	320 µM
$NiSO_4 \bullet 6\ H_2O$	0.1	Sigma	N4882	380 µM
$CuCl_2 \bullet 2H_2O$	0.002	Sigma	307483	10 µM
Na_2SeO_3	0.006	Sigma	S-1382	30 µM
$Na_2WO_4 \bullet 2H_2O$	0.008	Fisher	S450-500	20 µM



LS medium has a longer lag time and was more variable then either the LS3D medium or LS4D. LS4D proved to be more reproducible then either of the other media. Baars medium (data not shwn) had a shorter lag but did not reach as high a density as LS4D or even LS medium.

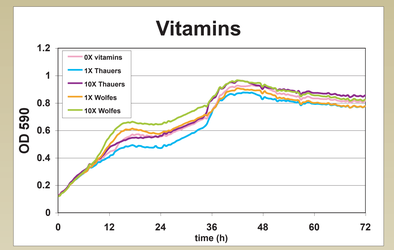


A variety of reducing agents were tested for reducing capability without toxicity to *D. vulgaris*. Titanium (III) citrate promotes the fastest growth of *D. vulgaris* without inhibiting final cell densities. Optimal growth resulted from TiCi at 0.5%

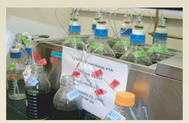


A 1% inoculum from a –80°C stock yields a log phase culture in 72 hours
A 1% inoculum of a log phase culture yields a log phase culture in 48 hours
A 10% inoculum of a log phase culture yields a log phase culture in 24 hours
Larger initial inoculum allows you to reduce the amount of reductant and which decreases the lag time.

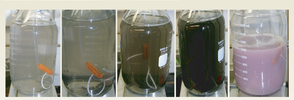
Note: Lactate concentrations and sulfate cocentrations were also tested. Lactate and Sulfate reduced the maximum density at concentrations below 10 mM. Yeast concentrations below 0.05% reduced the growth rate only slightly.

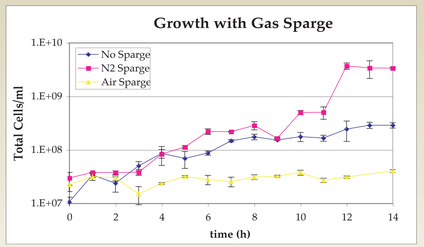


Comparison of the effects of Thauers vs Wolfes vitamins on the growth of *D. vulgaris* in LS4D. Vitamin composition and concentration resulted in little difference in growth. Thauers vitamins contain choline and 10 times more vitamin B12 than Wolfes.



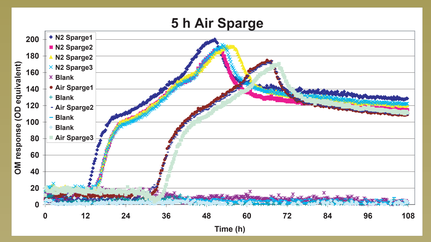
Oxidative stress occurs in anaerobic sediments where *Desulfovibrio* is widely distributed as oxygenated groundwater percolates through the sediments. Free oxygen radicals cause protein, membrane and DNA damage in cells. Batch cultures of *D. vulgaris* were oxygen stressed by sparging log phase cultures with oxygen as air for 5 hours, 1 generation time.



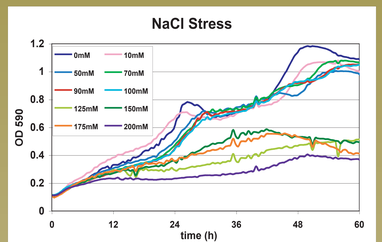


Live/Dead cell staining after sparging reveals the ratios of live to dead cells after this stress event. Prior to sparging 90 % of the cells were live. After 5h of oxygen sparge approximately 7% of the cells were live, as compared to the nitrogen sparged control culture in which 70% were live.

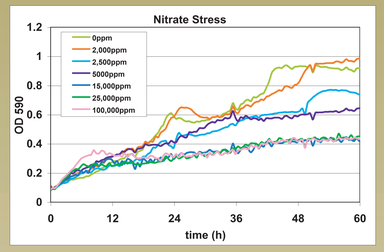
Stressors (MIC)



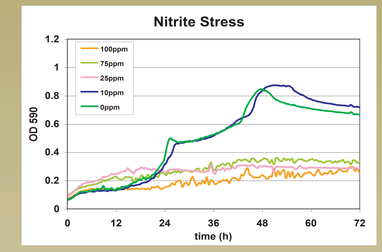
During the oxidation event no growth in the oxygen sparged culture occurs. However, upon completion of the sparge event, *D. vulgaris* cultures are capable of continued growth. Note: MIC is defined here as the minimum concentration that inhibits growth. See ASM poster N-152 and I-095 for related studies on air stress.



Soil microorganisms are challenged by osmotic stress as highly saline environments occur from weathering processes. Several soil microbes have the capability of adapting to high saline environments using regulatory pumps. Osmotic stress as NaCl has no effect of *D. vulgaris* growth up to 100 mM NaCl. Growth of *D. vulgaris* is retarded in the range of 150 mM NaCl, and inhibited at 200 mM NaCl. See ASM Poster K-072 for transcriptional analyses.



Nitrate is a common contaminant of sediment and groundwater at DOE sites. Although nitrate serves as an electron acceptor in microbial respiration, it is generally inhibitory at concentrations above 100 ppm. Retarded growth of *D. vulgaris* in LS4D is seen with concentrations in the range of 2,500 ppm NO_3. There is no effect on growth with 2,000 ppm and complete inhibition with 15,000 ppm. Several gene and protein responses have been detected as a stress response to elevated concentrations of nitrate (See ASM Poster K-081).



In stressed environments denitrification, a 2 step process, may not be complete. Nitrite accumulation can occur in these environments as a result. Nitrite is inhibitory at significantly lower concentrations than nitrate. In addition, SRBs show increased susceptibility to the toxic effects of nitrite (see ASM Poster K-081). Growth of *D. vulgaris* in LS4D is not affected with 10 ppm NO_2 and is inhibitory at concentrations above 25 ppm.

Conclusions

1. **LS4D is a defined medium that provide highly reproducible results for stress studies using automated high-throughput culture techniques for sulfate reducing bacteria.**
2. **Using a 10% inoculum and no reductant *D. vulgaris* has a 5 h gereration time and no lag phase.**
3. ***D. vulgaris* was able to survive more than 5 h of sparging with air and regrow anaerobically with a 10-12 h lag.**
4. **Sparging of *D. vulgaris* with N_2 gas had no effect on growth rate but it did allow the culture to reach a higher density.**
5. ***D. vulgaris* MIC for NaCl was 150 mM.**
6. ***D. vulgaris* MIC for Nitrate was 2500 ppm.**
7. ***D. vulgaris* MIC for Nitrite was 10 ppm.**

See ASM Posters I-051, I-095, N-345, K-072, K-077, K-081, N-061, N-152, Q-142, Q-155, and Q-373 for related studies.

References

Aranki, A., and R. Freter. 1972. Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. American Journal of Clinical Nutrition 25:1329-1334.
Jones, G. A., and M.D. Pickard. 1980. Effect of titanium(III) citrate as a reducing agent on growth of rumen bacteria. Appl. Environ. Microbiol. 39:1144-1147.
Philipp, G., R. Murray, W. Wood, and N. Krieg. 1994. Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, DC. 791 pp.
Brandis, A., and R. K. Thauer. 1981. Growth of Desulfovibrio species on hydrogen and sulfate as sole energy source. J. Gen Microbiol. 126:249-252.

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